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TITLE: Epigenetic Mediation of Endocrine and Immune Response in an Animal Model of Gulf War Illness

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14. ABSTRACT

There are now compelling human epidemiological and animal experimental data that indicate the risk of developing complex diseases is influenced by persistent epigenetic adaptations in response to environmental exposures such as toxins and stress. We propose to examine the epigenomic response to diisopropyl fluorophosphates (DFP), a sarin surrogate, and associated changes to the immune and endocrine response to lipopolysaccharide (LPS) challenge in a mouse model of Gulf War Illness (GWI), with stress hormone exposure as an experimental mediator. We will study the relationship between changes in DNA methylation and chromatin modifications in peripheral blood and the brain (specifically hippocampus and prefrontal cortex) in order to pursue a mechanistic understanding of the underlying pathology of GWI. During this reporting period, we have begun data collection on DNA methylation modifications and gene expression profiles in the brains of mice exposed to saline control, corticosterone and DFP, and have developed and refined protocols for this project in line with the GWIRC sister project.

15. SUBJECT TERMS

Hypothalamic-Pituitary-Adrenal (HPA) axis, epigenetic, hippocampus, prefrontal cortex, lipopolysaccharide (LPS), diisopropyl fluorophosphate (DFP), corticosterone (CORT), peripheral blood mononuclear cell (PBMC), DNA methylation, Histone acetylation, immune system, genomics, mouse model

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Introduction

Epigenetic changes, including modifications to chromatin structure and DNA methylation, alter gene expression and cellular phenotype in the absence of variation in DNA sequence. There are now compelling human epidemiological and animal experimental data that indicate the risk of developing complex diseases is influenced by persistent epigenetic adaptations in response to environmental exposures such as toxins and stress. We propose to examine the epigenomic response to diisopropyl fluorophosphates (DFP), a sarin surrogate, and associated changes to the immune and endocrine response to lipopolysaccharide (LPS) challenge in a mouse model of Gulf War Illness (GWI), with stress hormone exposure as an experimental mediator. We will study the relationship between changes in DNA methylation and chromatin modifications in peripheral blood and the brain (specifically hippocampus and prefrontal cortex) in order to pursue a mechanistic understanding of the underlying pathology of GWI. Epigenetic profiles indicative of transcriptional enhancement or repression may help identify stable biomarkers of GWI related to exposure across a variety of environmental conditions. Because epigenetic marks are potentially reversible, elucidating the manner in which environmental interventions including immune-endocrine stressors alter epigenetic marks across the blood-brain barrier in the context of toxin exposure will offer insight into mechanisms leading to stable disease states and novel routes to therapeutic intervention. The overall objective of this project is to identify epigenetic mechanisms of altered Hypothalamic-Pituitary-Adrenal (HPA) axis and immune signaling in a mouse model of environmental exposures linked to GWI. DNA methylation and histone modifications will be examined in peripheral blood and the brain using a high-throughput genome-wide approach. This proposal adds substantial value to a funded GWIRP Consortium project designed to examine gene regulatory dynamics in a model of toxicant exposure (DFP, a sarin surrogate) and stress/immune challenge (CORT, LPS).

Keywords

Gulf War Illness, Hypothalamic-Pituitary-Adrenal (HPA) axis, epigenetic, hippocampus, prefrontal cortex, lipopolysaccharide (LPS), diisopropyl fluorophosphate (DFP), corticosterone (CORT), peripheral blood mononuclear cell (PBMC), DNA methylation, Histone acetylation, immune system, genomics, mouse model

Accomplishments

What were the major goals of the project?

The major goals of the project and associated milestones (month identified for completion/actual completion dates/percent completed) as stated in the approved Statement of Work (SOW) were:

Task 1: Validate mouse model and perform manipulations of animal subjects.

- Milestone: IACUC/ACURO Approval. (**month 3/December 2014**, **2015/100%)**

Task 2: Extraction of tissue samples and blood sample processing.

- Milestone: Validation of manipulation and collection of tissue samples (**Month 3/June 2015/40%**).
- Milestone: DNA from blood prepared for Sequencing (month 5/not yet completed)
- Milesone: DNA library preparation for sequencing of blood (month 9/not yet completed)
- Milestone: Blood epigenetic paper (month 18/not yet completed)

Task 3: Extraction of tissue samples and brain sample processing.

- Milestone: DNA from brain prepared for Sequencing (**month 8/Sept 2015/25%**)
- Milestone: DNA library preparation for sequencing of brain (**month 10/Sept 2015/25%**)
- Milestone: Brain epigenetic paper: (month 20/not yet completed)

Task 4: Gene expression analysis on blood and brain

- Milestone: Gene expression profiles for blood, hippocampus and prefrontal cortex (**month 9/Sept 2015/5%)**

Task 5: Analysis of epigenetics and gene expression across tissue types

- Milestone: Identify epigenetic correspondence across tissue types (month 28/not yet completed)
- Milestone: Functional epigenetics paper (month 36/not yet completed).

What was accomplished under these goals?

1) The specific objectives described in the approved SOW were:

Specific Aim 1: Assess epigenetic changes in peripheral blood in response to DFP exposure concomitant with sustained corticosterone stress hormone administration and subsequent antigen (LPS) challenge

Specific Aim 2: Assess epigenetic changes in brain as a function of DFP exposure, corticosterone administration and in response to LPS challenge

Specific Aim 3: Assess the relationship between epigenetic changes and gene regulatory dynamics in brain and in blood, and their relationship to transcriptomic and physiological outcomes

2) Major Activities and results during this reporting period:

Site visits: We have made site visits to review protocols and preliminary data to ensure alignment with the overarching Consortium GW120045 (project W81XWH-12-2-0085; Morris, PI).

Feb. 24-27 2015. Dr. Broderick met with Drs. O'Callaghan and Miller at CDC/NIOSH in Morgantown, WV:

- Delivered talk to NIOSH leadership and staff
- Discussed numerical analysis protocol with laboratory research staff, specifically the alignment of time course experiments between the current award and sisters projects conducted concurrently under the broader programmatic envelope of the GWI Consortium.
- Reviewed existing preliminary data, including initial whole genome profiling short-term (6-hour post-exposure) response to DFP, LPS and corticosterone (CORT) in cortex and hippocampus conducted with the Illumina mouse microarray.
- Coordinated sharing of existing RNA sequencing data with McGowan group for first review of most promising target genes

Mar. 30-31 2015. Dr. Broderick met with Dr. McGowan at University of Toronto. The visit consisted of an onsite review of animal experimental protocols being used under overarching project W81XWH-12-2-0085 with laboratory staff, specifically the timeline for dosing and exposure protocols proposed for GWIRC studies 1 and 2.

- Delivered a Department seminar describing aspect of the proposed numerical protocol

August 17-21 2015. Dr. Broderick met again with Drs. O'Callaghan and Miller at CDC/NIOSH in Morgantown, WV. Focus of this meeting was a through review of pathway projection methods and analysis of available data, in particular Illumina array profiling gene expression in astrocytes using a novel quenching and cell separation technique (BAC-TRAP)

Animal dosing: As stated in the goal for **Task 1**, we performed baseline and challenge studies on the animal

models, followed by animal sacrifice. We have revised our 'n' in order to determine variability in methylation status across conditions (see Bisulfite pyrosequencing, below and **Table 1** below for dosing schedule). Tissue samples, consisting of blood and brain from 44 animals were shipped from the CDC site (Dr. O'Callaghan) to UofT (Dr. McGowan) on June 24th 2015. This group was composed of 8 saline treated animals (T0 control), 8 CORT treated animals (T1 condition), 15 DFP treated animals (T2.1 condition) and 13 DFP+CORT treated animals (T2.2 condition). Mice were treated with CORT (200 mg/L 0.6% EtOH) for 4 days in the drinking water. At day 5, mice were treated with DFP (3.0 mg/kg s.c.) and sacrifice 6 hours later.

Table 1. Animal dosing schedule:

Groups	4 Day Treatment	Time Points	
		Day 5 (sac -6 hours post dosing)	21 Day (sac – 6 hours post dosing)
1	Water	Saline (N=10) [8 received]	-
2	CORT	Saline (N=10) [8 received]	-
3	Water	DFP (N=10) [15 received]	Saline (N=10)
4	CORT	DFP (N=10) [13 received]	Saline (N=10)
5	Water	DFP -	LPS (N=10)
6	CORT	Saline -	LPS (N=10)
7	CORT	DFP -	LPS (N=10)
8	Water	Saline -	LPS (N=10)
		Total	100

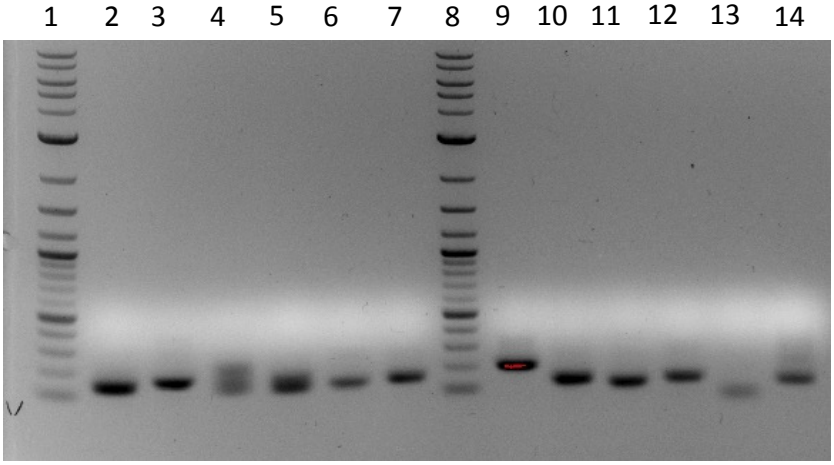
Trainee recruitment for molecular assays: We have recruited Dr. Benjamin Hing, postdoctoral fellow, Ms. Christine Lum, graduate student, and Mr. Shathveekan Sivanathan, technician, to U of T.

Blood and brain extractions: For **Task 2**, blood and brain samples were extracted and shipped from CDC to U of T. PBMC extraction was problematic from these samples, as cell separation was not ideal and thus few/no PBMCs were obtained from some samples, likely to due to shipping delays we encountered. As a result, RNA and DNA yield from PBMCs were inconsistent between samples.

Nucleotide extractions, validation and high throughput sequencing: For **Tasks 3 an 4,** we succeeded in harvesting the brain tissues (hippocampus and prefrontal cortex) described above (see Animal dosing) and have extracted good quality RNA and DNA from each of the samples above. We have made progress in DNA methylation and histone modification procedures in the following areas:

Bisulfite-pyrosequencing for validation of DNA methylation modifications at specific loci: It is presently unknown how variable methylation changes are between treatment groups for specific genes. This would have an impact on the number of samples required to observe robust DNA methylation changes between treatment groups by Reduced Representation Bisulfite-Sequencing (RRBS). To address this issue, we have designed bisulfite-pyrosequencing primer to validate DNA methylation changes in genes that are known to have altered gene expression levels in the mouse model of gulf war syndrome (O'Callaghan et al., J Neurochem, 2015). The genes are TNF-α, CCL, IL1-β, LIF, IL6 and OSM. Primers were designed against promoter and proximal regulatory regions defined by acetylation of histone 3 lysine 27 (H3K27ac) which contained MspI restriction site (CCGG) and putative GR binding sites so that data obtained could be compared to RRBS data, which also uses MspI digested DNA for library preparation. PCR conditions have been optimised for these primer sets (see **figures 1-2** below and **Appendix 1** for prelminary data). This will be followed by pyrosequencing validation of the primer sets and profiling of the samples for each brain region. The validation is thus being done in parallel with high-throughput sequencing to maximize the efficiency of our experiments.

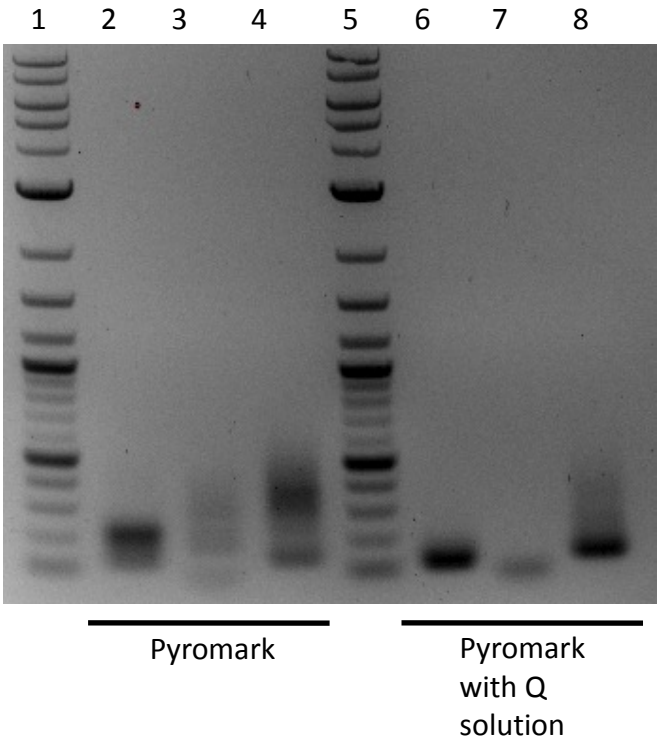
Figure 1. Optimization of primer assays for Bisulfite Pyrosequencing: Part 1.



lane 1 - 2 log ladder
lane2 - TNF promoter
lane3 - TNF Intron
lane 4 - CCLprom
lane 5 - CCL enhancer
lane 6 - ILB promoter 1
lane 7 - ILB promoter 2
lane 8 - 2 log ladder
lane 9 - LIF promoter 1
lane 10 - LIF promoter 2
lane 11 - LIF enhancer
lane 12 - IL6 promoter
lane 13 - OSM promoter
lanen 14 - OSM intron

Most genes show single bands indicating specific products. We have redone CCLprom (lane 4) with pyromark PCR and determine if pyromark PCR might improve OSM promoter (lane 13) and OSM intron (lane 14).

Figure 2. Optimization of primer assays for Bisulfite Pyrosequencing: Part 2.



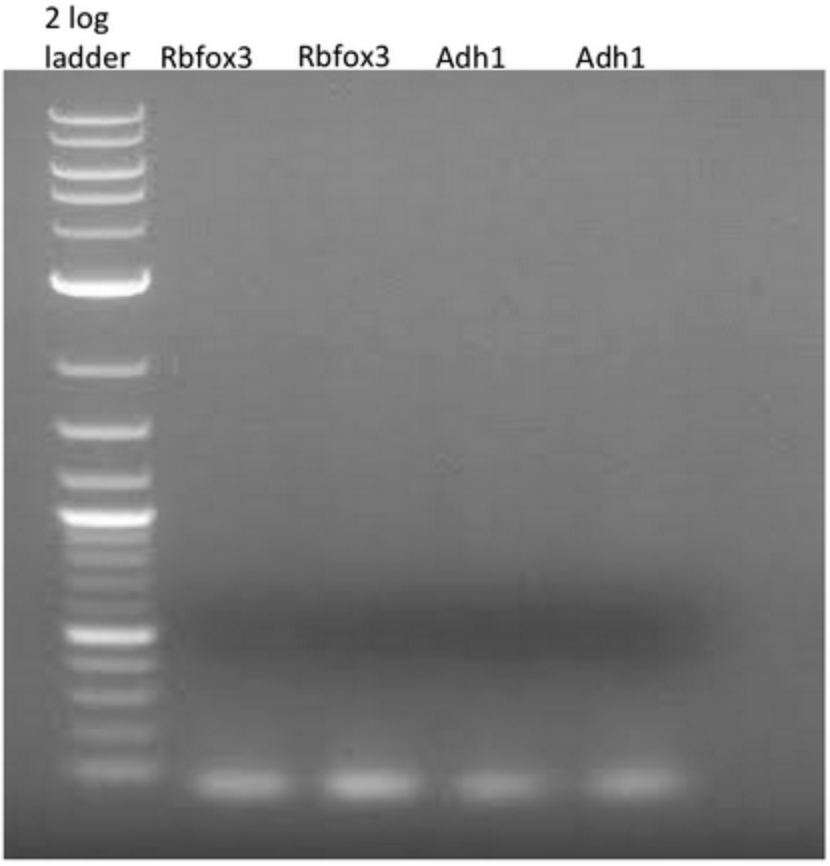
Lane 1 - 2 log ladderLane 2 - CCLprom
Lane 3 - OSMprom
Lane 4 - OSM Intron
Lane 5 - 2 Log ladder
Lane 6 - CCL prom with Q solution
Lane 7 - OSMprom with Q solution
Lane 8 - OSM Intron with Q solution

These results suggest that the addition of Q solution improves the specificity of the PCR for CCL promoter (Lane 6), OSM promoter (Lane 7), and OSM intron (Lane 8).

Reduced Representation Bisulfite Sequencing (RRBS) for genome-wide DNA methylation modification analysis: We have established logistic capabilities to carry out a novel one-day RRBS protocol at no additional cost to the grant, which would provide high-throughput library preperation of the samples and dramatically accelerate our objectives of preparing libraries (from an initially planned 1 week procedure). We have completed library prep of 8 samples consisting of 4 saline controls (T1) and 4 DFP+CORT (T2.2) samples in order to test our protocol. One of the saline controls will be performed in triplicate to determine technical variation of the assay. We are pooling 10 samples in a lane which should provide sufficient depth of coverage (10x) to detect at least a 10 % change in DNA methylation level. We have sent samples for DNA sequencing and are presently awaiting results from the core facility.

Chromatin Immunprecipitation (ChIP) sequencing for genome-wide histone modifications: The ChIP protocol is currently in the optimization stage. We have first targeted H3K27ac, which is a histone modification mark of active regulatory regions. To validate the assay, primers were designed against alcohol dehyrogenase promoter (Adh1) and Rbfox3 which are genes expressed in the liver and in the brain, respectively. During validation of the ChIP-seq protocol, the signal produced from the pulldown by the target antibody was higher than that of the iso-type antibody showing target specificity of the target antibody. However, signal strength between Adh1 and Rbfox3 control genes were the same in the brain, which deviates from the data observed in the UCSC genomic database. Although melt curve of SYBR green qPCR showed single melt curve suggesting single amplified product. Product size was however bigger than expected suggesting off-target amplification. Using a different SYBR green mastermix (see **Figure 3** below and **Appendix 3** for preliminary data), size of the amplicon is now as expected. Another pulldown is required to determine if the change in SYBR green mastermix has resolved the issue.

Figure 3. Validation of PCR for ChIP protocol with Adh1 and Rbfox3 control genes



These data show single bands in each experimental well, indicating a specific product has been obtained of the correct size for each gene and that the PCR conditions have been optimized.

Nanostring quantitative transcript abundance analysis: We have initiated the studies described in **Task 4**. After

thorough review of human data available through the sister project and available nanostring kits for mouse, we have decided not to use a custom kit. Instead, we have selected the nCounter Mouse Immunologymmunology kit which provides a good coverage of pathways identified through work with our human samples at a much lower price. We have started QC runs using this chip (Dr. Nathanson, NSU).

RNA sequencing: We have obtained Illumina RNA sequencing data from first exposure conditions (saline, CORT, CORT+DFP) available from the CDC database as a result of the sister project (see GWIRC annual report). We have begun to review these data to and have made adjustments to our basic software platform to broaden array of analyses possible with our epigenetic data.

Fluorescence-assisted Cell Sorting (FACS) of neurons and glia from brain tissue: DNA methylation is known to differ between neurons and glial cells (Iwamoto et al., 2011; Kozlenkov, NAR, 2014), the primary cell types in the brain. Work by our Dr. O'Callaghan (Co-PI on this project) has indicated that glia cells may drive changes in expression associated with the exposures used in this project. We conducted a pilot feasibility assessment using cell separation by Fluorescence-assisted cell sorting (FACS) of neural tissue into neurons and glia. The figures below show the results of FACs analysis of samples consisting of an n = 3 of a mix group 1 hippocampus and 2 cortices. FACs showed two peaks: A group with low level of NeuN staining and a group with a high level of NeuN staining. The NeuN group with low staining constituted ~25% of stained nuclei whereas NeuN group with high staining constituted ~70% of stained nuclei (**Figure 4** below). This is consistent with known proportion of glial cells to neurons ratio in brain regions such as the cortex. Studies have demonstrated that certain glial cells also express NeuN (Darlington et al., 2008), suggesting that the lowly stained nuclei are glial cells. Furthermore, as shown below, bisulfite pyrosequencing showed significantly higher methylation level in GFAP promoter for the highly stained nucleus compared to low stained nucleus further showing that highly stained NeuN nuclei are neurons and low stained nuclei are glial cells (**Figure 5** below). As such, the current data show that the FACs method is optimized for subsequent use to interrogate the epigenetics of neurons and glia.

Figure 4. Cytometry with NeuN staining showing two groups of cells, assumed to be neurons and glia.

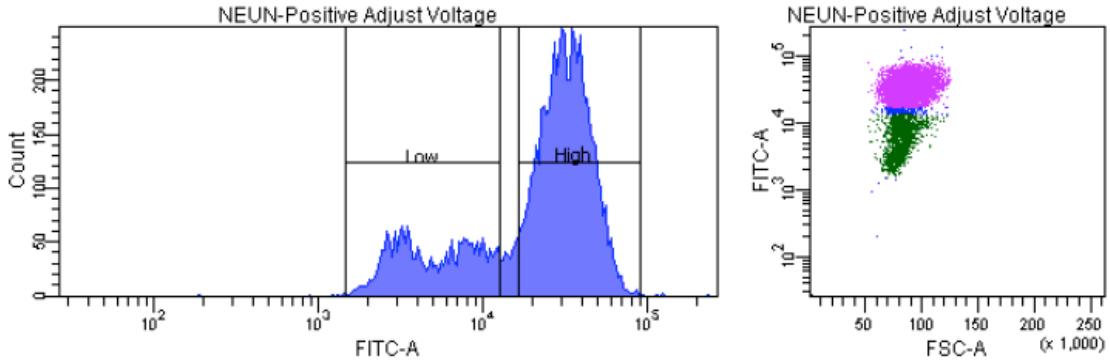
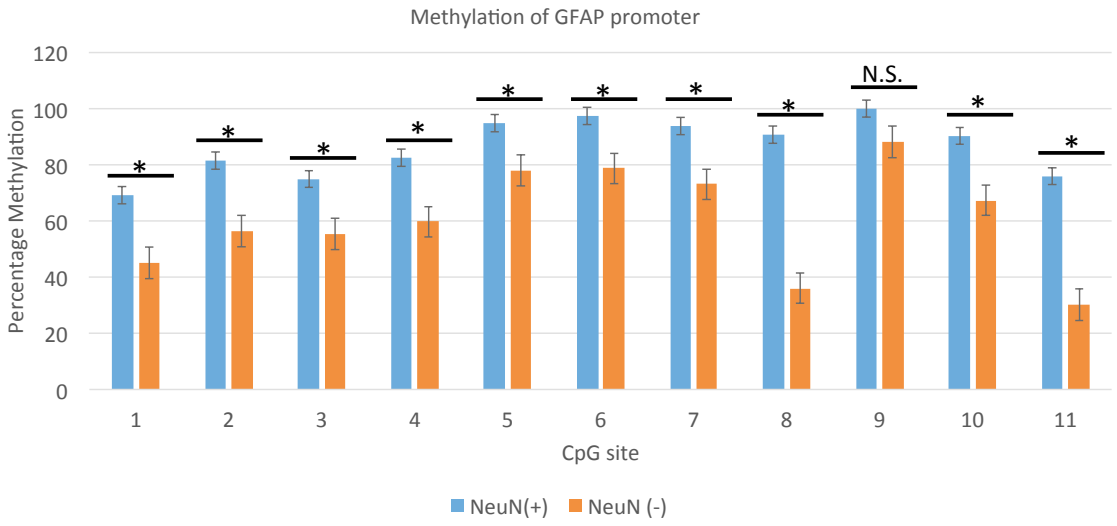


Figure 5. Bisulfite Pyrosequencing of DNA methylation in the GFAP promoter of NeuN+ and NeuN- cells separated by FACs, confirming neuron and glia status respectively.



Statistical analyses: We recruited our research associate, Patrick Gourdet, into Dr. Broderick's group at NSU in late December 2014. Mr Gourdet has a background in computer science and we have been training him specifically on the use of the MatLab suite of bioinformatics tools and in the principles of high dimensional statistics as they apply to gene expression. We have also been training him on the algorithmic details of the pathway analysis technique proposed by Efroni et al. (2007).

What opportunities for training and professional development has the project provided?

This project has provided technical training for postdoctoral fellow Dr. Benjamin Hing, graduate student Christine Lum and technician Shathveekan Sivanathan at UofT in laboratory activities related to the project during this reporting period. In addition, we recruited research associate Patrick Gourdet to Nova Southeastern University (NSU) and have been training him in high-dimensional data analysis including outlier detection and regression using projection methods such as Principal Component Regression (PCR) and Partial Least Squares (PLS).

How were the results disseminated to communities of interest?

Some of our preliminary results were shared in reporting for the GWIRC. We have been refining our analytical protocol in collaboration with DoD with human data obtained through the sister project, and plan to apply this to the present award. Specifically, we have started pathway projection analysis on mouse data, and have completed a preliminary analysis of mouse DFP+CORT, DFP, CORT, and saline in cortex and hippocampus using Illumina RNA-seq data. We have begun to refine the resolution of the data related to quality control and pathway techniques to adjust from the highly variable human condition to the mouse condition consisting of smaller groups. Our techniques are being refined in collaboration Dr. Jacques Reifman of DoD. This will enable us to use this refinement for network analysis with epigenetic data in our mouse model.

What do you plan to do during the next reporting period to accomplish the goals?

During the next reporting period, we plan to obtain LPS-treated brain and blood samples (T3 condition) and perform DNA sequencing for DNA methylation modifications for brain-PFC and blood samples. We will perform pyrosequencing validation of the primer sets for the genes TNF-α, CCL, IL1-β, LIF, IL6 and OSM within each brain region. The validation is thus being done in parallel with high-throughput sequencing to maximize the efficiency of our experiments. We will be able to take advantage of existing RNA-sequencing

data to directly compare them with the epigenetic profiles we will obtain from the current work.
We also plan to have optimized our ChIP-seq protocol and to perform ChIP-sequencing for histone modifications on brain and blood. We plan to perform QC analysis of sequencing data and to analyze RRBS and ChIP-seq comparisons between groups. Finally, we are continuing our feasibility analysis of FACS sorted neurons and glia to determine whether enough material for epigenetic assessments in glia can be made from each tissue source (cortex, hippocampus).

Impact

What was the impact on the development of the principal discipline(s) of the project?

The techniques we developed will serve our project as well as other DoD projects. Please see section on 'results dissemination' above for a description of the technical refinement of our analytical approach.

What was the impact on other disciplines?

This work feeds back into our thinking of how certain gene regulatory circuits might fail in sister projects under the broader umbrella of the consortium.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

This reporting period (September 2, 2015), McGowan was invited to speak about his research program at the US Environmental Protection Agency (EPA) workshop on Epigenetics and Cumulative Risk. The goal of this workshop was "to explore and lay groundwork for practical application of epigenetic data in assessment of cumulative risk from multiple environmental factors." The results of this project will thus likely impact public knowledge, funding and policy decisions in environmental health more broadly.

Changes/Problems

Changes in approach and reasons for change

As mentioned above, we have continued to refine our animal dosing schedule (number of subjects), methodological decisions (NanoString platform) data analysis platform in light of data generated from the GWIRC project in progress, at no additional cost to the current project. In addition, we have piloted a protocol to enable cell separation by Fluorescence-assisted cell sorting (FACS) of neural tissue into neurons and glia (see Section 2 of 'Major Activities' above for results).

Actual or anticipated problems or delays and actions or plans to resolve them

We encountered an unexpected delay in the trans-border shipment of samples from CDC to UofT. This delay affected the quality and quantity of PBMCs we were able to harvest from the blood samples, with too little DNA for many samples for downstream analyses. We plan to extract PBMCs at the CDC site prior to shipping them to UofT during the next reporting period. The samples will be frozen on dry ice for shipment.

During validation of the ChIP-seq protocol (**Appendix 2**), the signal produced from the pulldown by the target antibody was higher than that of the iso-type antibody showing target specificity of the target antibody.

However, signal strength between Adh1 and Rbfox3 control genes were the same in the brain, which deviates from the data observed in the UCSC genomic database. Although melt curve of SYBR green qPCR showed single melt curve suggesting single amplified product. Product size was however bigger than expected suggesting off-target amplification. Using a different SYBR green mastermix (see **Appendix 3** for preliminary data), size of the amplicon is now as expected. Another pulldown is required to determine if the change is SYBR green mastermix has resolved the issue.

Changes that had a significant impact on expenditures

Due to visa delays, Dr. Benjamin Hing, the postdoctoral fellow at UofT was not able to start until January 2015.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

Products

Nothing to report.

Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name:	Patrick McGowan, PhD
Project Role:	Principal-Investigator (NSU site PI)
Research Identifier:	none
Nearest person month worked:	3
Contribution to Project:	Lead PI overseeing the project. Direct supervision of molecular biology studies related to epigenetics data.
Funding Support:	

Name:	Gordon Broderick, PhD
Project Role:	Co-Investigator (NSU site PI)

Research Identifier:	eCommons: gbroderick
Nearest person month worked:	0.36
Contribution to Project:	Head of computational biology. Has worked on the computational models for animal and human research to assist in protocols and findings.
Funding Support:	NIH, VA

Name:	Mariana Morris, PhD
Project Role:	Co-Investigator
Research Identifier:	eCommons: mariana
Nearest person month worked:	0.36
Contribution to Project:	Overseeing the animal protocols and in charge of the anima research. Contributes technical expertise in animal experimentation. PI of GWIRC project
Funding Support:	NIH

Name:	Benjamin Hing, PhD
Project Role:	Postdoctoral fellow
Research Identifier:	None.
Nearest person month worked:	9
Contribution to Project:	Active in epigenetic assays
Funding Support:	

Name:	Ms. Christine Lum
Project Role:	Graduate student
Research Identifier:	None
Nearest person month worked:	9
Contribution to Project:	Active in epigenetic assays
Funding Support:	

Name:	Mr. Shathveekan Sivanathan
Project Role:	Technician
Research Identifier:	None
Nearest person month worked:	1
Contribution to Project:	Assists postdoctoral fellow and graduate student with epigenetic assays
Funding Support:	

Name:	Mr. Patrick Gourdet
Project Role:	Research Programmer
Research Identifier:	None
Nearest person month worked:	10
Contribution to Project:	Active in data analysis and software platform design
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Name:	Centers for Disease Control and Prevention National Institute for Occupational Safety and Health
Location:	1095 Willowdale Road Morgantown, WV 26505
Contribution:	Chemical toxicology project collaboration
Financial:	None
In-kind Support:	None
Facilities:	None
Collaboration:	Partner's staff works with project staff in the project.
Personnel Exchanges:	None
Other:	None

Name:	Nova Southeastern University
Location:	Institute for Neuro-Immune Medicine University Park Plaza 3440 South University Drive Fort Lauderdale, FL 33328
Contribution:	Genomic profiling and computational analyses
Financial:	None
In-kind Support:	None
Facilities:	None
Collaboration:	Partner's staff works with project staff in the project.
Personnel Exchanges:	None
Other:	None

Other.

Nothing to report.

Special Reporting Requirements

Nothing to report.

Appendices

Appendix 1. Bifulfite Pyrosequencing Optimization for validation of specific genes.

Objective: Optimizing PCR step of bisulfite-pyrosequencing for primers designed around TNF, CCL, IL1B, LIF, IL6 and OSM (O'Callaghan et al., J. Neurochem 2015)
PCR was performed using Thermopol PCR

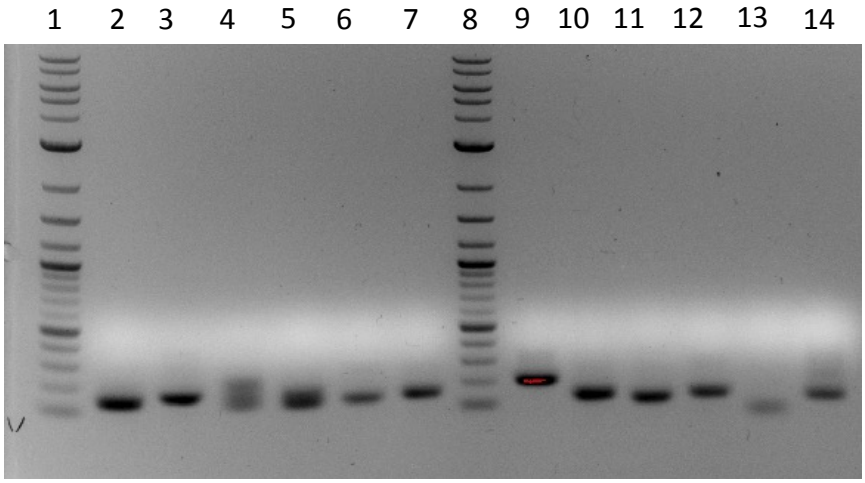
	Final conc.	µl	x7 (µl)
Buffer (10X)	1X	2.5	30
10mM dNTPs	200µM	0.5	6
Primer (5µM)	200nM	1	12
DNA		1	12
Taq		0.125	1.5
H2O		19.875	238.5
Total		25	300
PCR2			
	Final conc.	µl	x14 (µl)
Buffer (10X)	1X	2.5	35
10mM dNTPs	200µM	0.5	7
Primer (5µM)	200nM	1	14
DNA		2	28
Taq		0.125	1.75
H2O		18.875	264.25
Total		25	350

First PCR thermal cycling condition:

STEP	TEMP	TIME
Initial Denaturation	95°C	30 sec
30 Cycles	95°C	30 sec
	57°C	30 sec
	68°C	30 sec
Final Extension	68°C	5 minutes
Hold	4°C	

Second PCR thermal cycling condition:

STEP	TEMP	TIME
Initial Denaturation	95°C	30 sec
35 Cycles	95°C	30 sec
	53°C	30 sec
	68°C	30 sec
Final Extension	68°C	5 minutes
Hold	4°C	

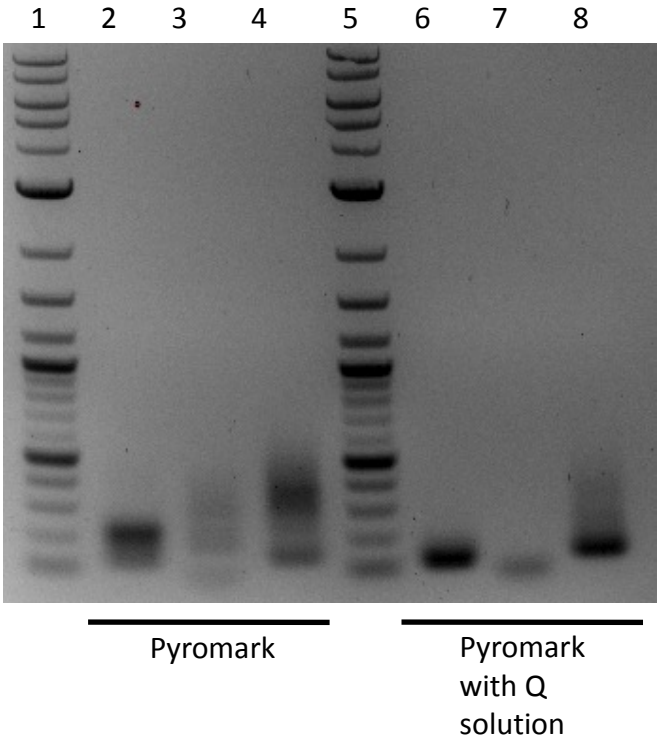


lane 1 - 2 log ladder
lane2 - TNF promoter
lane3 - TNF Intron
lane 4 - CCLprom
lane 5 - CCL enhancer
lane 6 - ILB promoter 1
lane 7 - ILB promoter 2
lane 8 - 2 log ladder
lane 9 - LIF promoter 1
lane 10 - LIF promoter 2
lane 11 - LIF enhancer
lane 12 - IL6 promoter
lane 13 - OSM promoter
lanen 14 - OSM intron

Redo CCLprom (lane 4) with pyromark PCR and determine if pyromark PCR might improve OSM promoter (lane 13) and OSM intron (lane 14)

PCR 1		with Q solution	without Q solution
	Final conc.	µl	µl
Master mix (2x)	1X	12.5	12.5
Coral load (10x)	1X	2.5	2.5
Q solution (5x)	1X	5	0
Primer (5µM)	200 nM	1	1
DNA		1	1
H2O		3	8
Total		25	25
PCR 2		with Q solution	without Q solution
	Final conc.	µl	µl
Master mix (2x)	1X	12.5	12.5
Coral load (10x)	1X	2.5	2.5
Q solution (5x)	1X	5	0
Primer	200 nM	1	1
DNA		2	2
H2O		2	7
Total		25	25

PCR 1		
Initial PCR activation step	95°C	15 min
30 Cycles	94°C	30 sec
	56°C	30 sec
	72°C	30 sec
Final Extension	72°C	10 min
Hold	4°C	
PCR 2		
Initial PCR activation step	95°C	15 min
45 Cycles	94°C	30 sec
	56°C	30 sec
	72°C	30 sec
Final Extension	72°C	10 min



Lane 1 - 2 log ladderLane 2 - CCLprom
Lane 3 - OSMprom
Lane 4 - OSM Intron
Lane 5 - 2 Log ladder
Lane 6 - CCL prom with Q solution
Lane 7 - OSMprom with Q solution
Lane 8 - OSM Intron with Q solution

Appendix 2. Native ChIP optimisation

MNase Titration

Objective: To determine length of time required for optimal MNase digestion.
Parameter: 50U of MNase used for reaction. Reaction was performed at 37°C for the length of time specified below.

	µl
Sample nuclei (PBS)	40
10X Mnase buffer	6
Sodium butyrate (5mM) (stock 100mM)	0
BSA (100ng/µl) (stock 6000ng/µl)	1
Mnase enzyme (25 U/µl)	2
H2O	11
Total	60

Make up of other reagents:

BSA

stock BSA	10mg/ml	10µg/µl
	10000ng/µl	
required 6000 ng/µl	1.6666667	
For 20µl	12	BSA (µl)
	8	H2O (µl)

MNase

	µl
Enzyme (2000U/µl)	1
BSA (6000ng/µl)	1.3333333
Buffer	8
H2O	69.666667
Total	80

1% deoxycholate/1%Triton X

4% deoxycholate	
2g in 50 ml	
	ml
1% Triton X and 1% deoxycholate	
Triton X	0.1
4% dexoycholate	2.5
H2O	7.4
total	10

EDTA

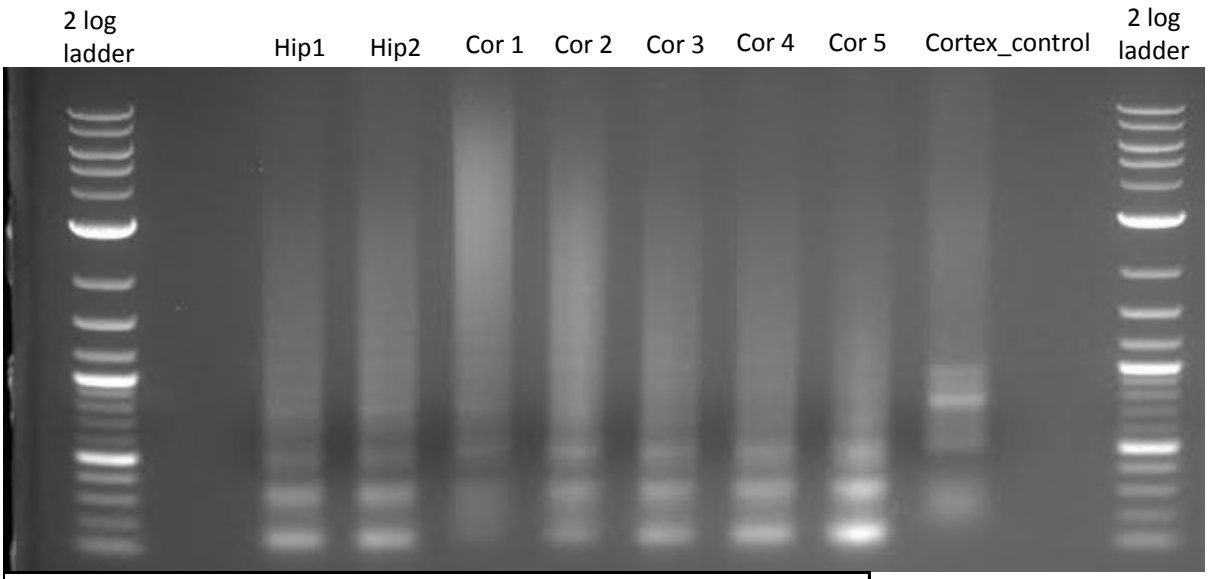
0.5M EDTA		
for 100mM	10	
For 1ml	200	µl of 0.5M EDTA
	800	µl H2O

Results

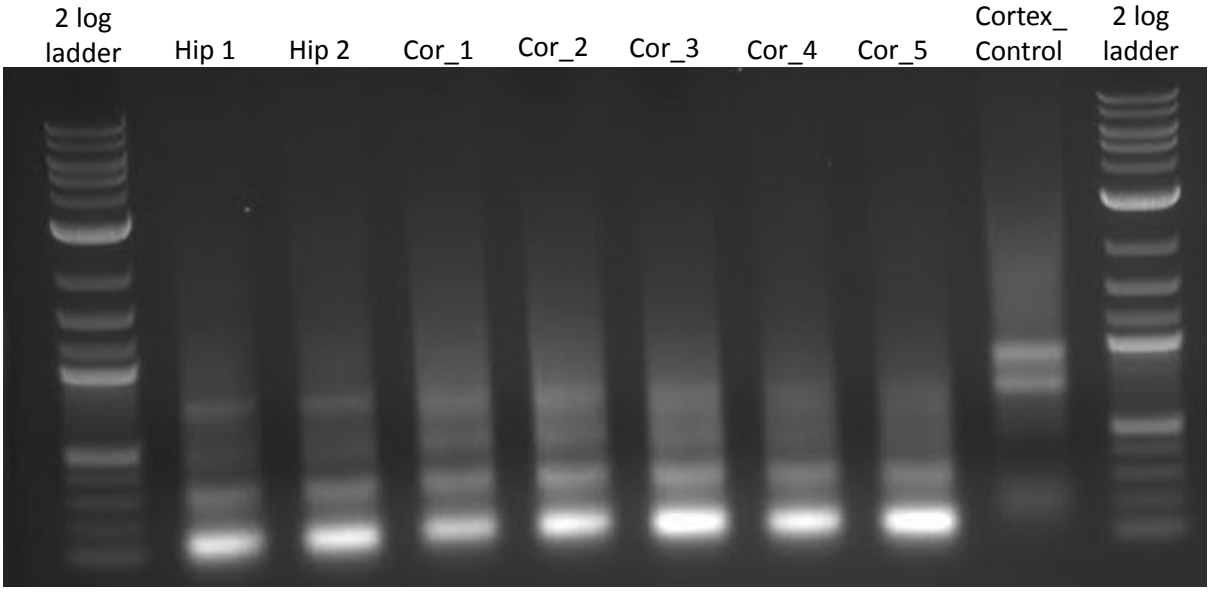
Experiment 1				
	ng/µl	260/280	260/230	digestion time (min)
Hippocampus 1	631.9	1.87	1.95	10
Hippocampus 2	969.3	1.79	1.98	10
Cortex_1	828.4	1.79	1.98	10
Cortex_2	888.3	1.74	1.96	10
Cortex_3	877.3	1.75	1.95	10
Cortex_4	959.5	1.73	1.91	10
Cortex control	631.9	1.93	1.89	0

- Experiment not run on gel

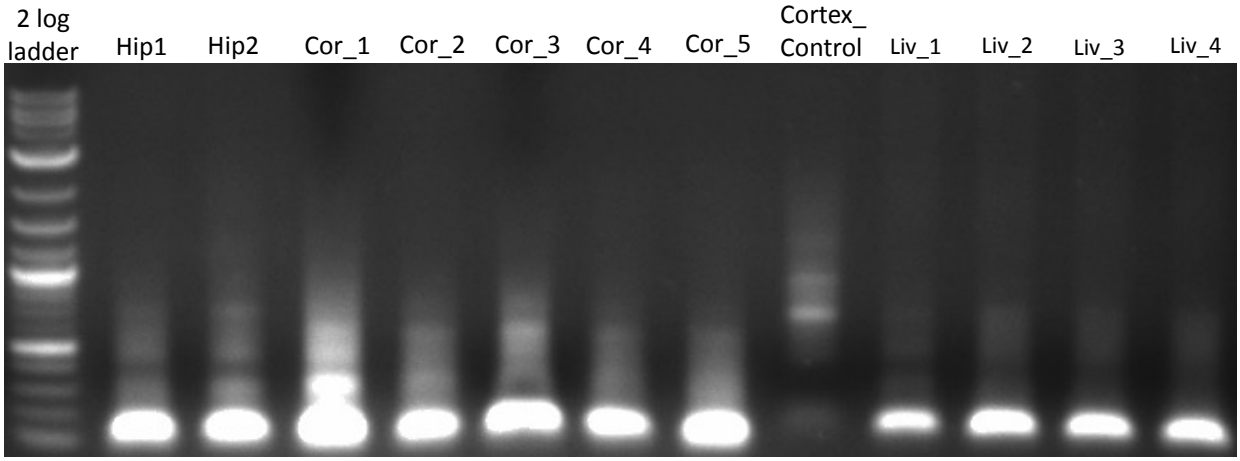
Experiment 2				
	ng/µl	260/280	260/230	digestion time (min)
Hippocampus 1	555.7	1.88	2.01	5
Hippocampus 2	596.7	1.87	2.03	5
Cortex_1	627.2	1.88	2.01	2
Cortex_2	728.6	1.84	2.06	4
Cortex_3	556.7	1.88	1.98	6
Cortex_4	588.7	1.88	1.98	8
Cortex _5	656.5	1.87	1.97	10
Cortex control	653.6	1.94	1.92	0

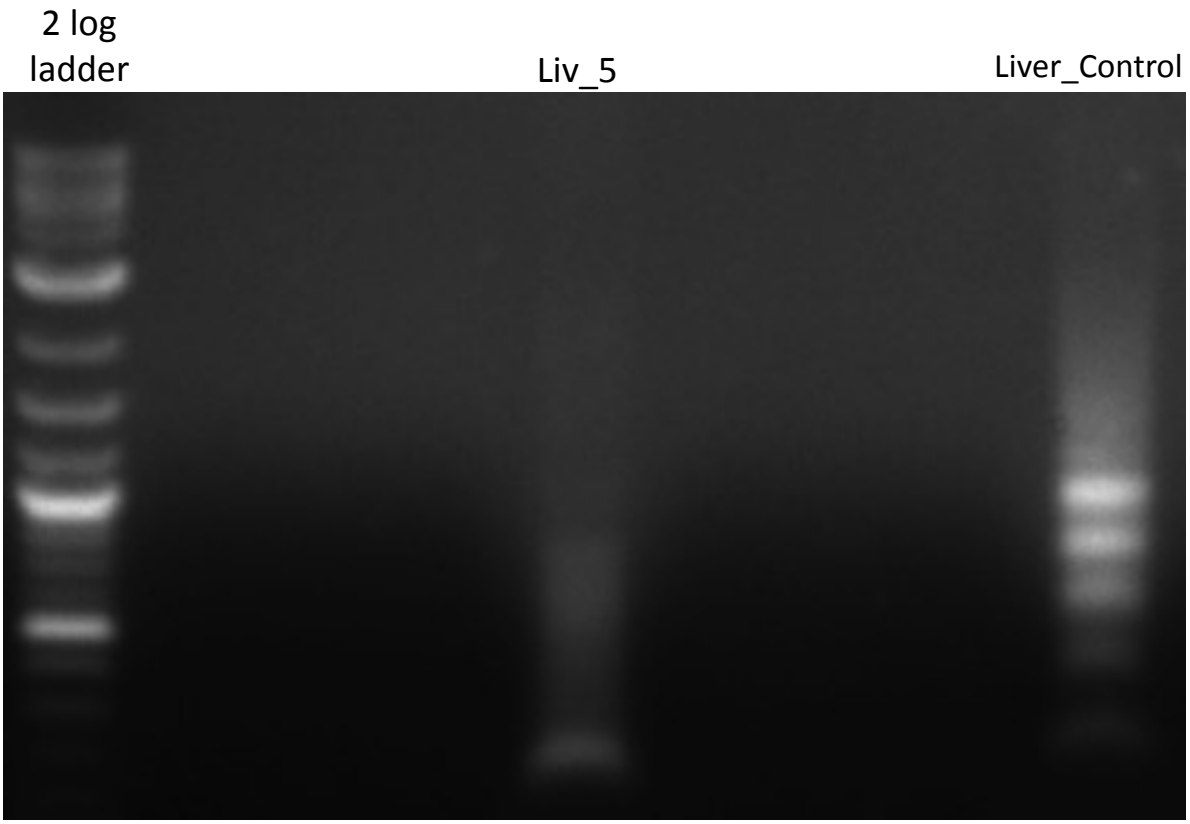


Experiment 3				
	ng/µl	260/280	260/230	digestion time (min)
Hippocampus 1	761.3	1.96	2.29	15
Hippocampus 2	800.2	1.94	2.25	15
Cortex_1	856.3	1.95	2.29	10
Cortex_2	983.8	1.98	2.29	12
Cortex_3	1038.2	1.97	2.17	15
Cortex_4	612.6	1.94	2.19	17
Cortex _5	850.4	1.96	2.23	20
Cortex control	851.7	2.08	2.19	0

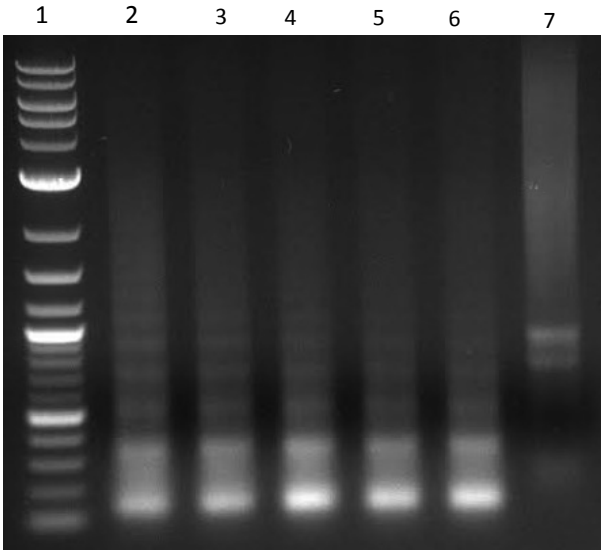


Experiment 4				
	ng/µl	260/280	260/230	digestion time (min)
Hippocampus 1	761.3	1.96	2.29	30
Hippocampus 2	800.2	1.94	2.25	30
Cortex_1	856.3	1.95	2.29	20
Cortex_2	983.8	1.98	2.29	25
Cortex_3	1038.2	1.97	2.17	30
Cortex_4	612.6	1.94	2.19	35
Cortex _5	850.4	1.96	2.23	40
Cortex control	851.7	2.08	2.19	0
Liver_1	1241.3	>1.8	>1.8	10
Liver_2	1749.8	>1.8	>1.8	20
Liver_3	1586.7	>1.8	>1.8	25
Liver_4	1615.5	>1.8	>1.8	30
Liver_5	1579.4	>1.8	>1.8	35
Liver_control	378.3	>1.8	>1.8	0

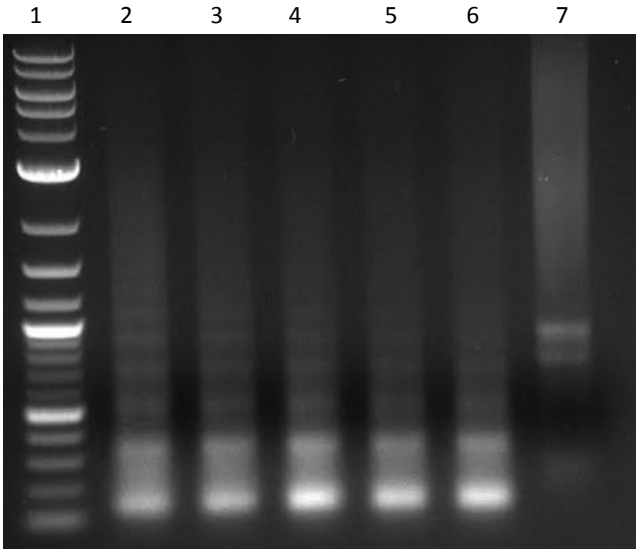




Conclusion:
20 min is optimal digestion time for Cortex and Hippocampus.



1. 2 log ladder
2. 2 min
3. 4 min
4. 6 min
5. 8 min
6. 10 min
7. Liver genomic DNA – uncut



1. 2 log ladder
2. 7 min
3. 10 min
4. 13 min
5. 15 min
6. 17 min
7. Liver genomic DNA – uncut

Objective: Determining optimal annealing temperature
10ng and 50ng of DNA used for amplification for SYBR green PCR. 200g of DNA used for Thermopol PCR amplification. Please note that the annealing temperature tested were 53°C, 55°C, 57°C, 60°C, 62°C, 64°C.

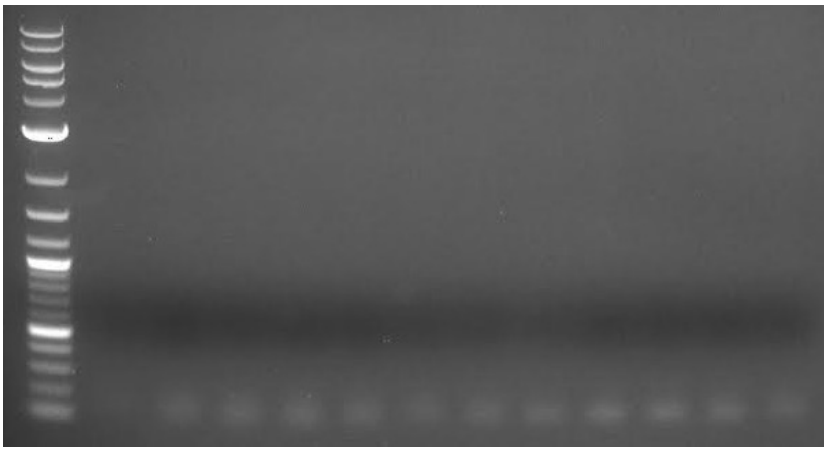
Fast SYBR green thermal cycling condition:

Select Fast Mode				
Instrument	**Step**	**Temperature (°C)**	**Duration**	**Cycles**
• Step One • StepOne Plus • 7500 Fast	AmpliTaq® Fast DNA Polymerase, UP Activation	95	20 sec	HOLD
	Denature	95	3 sec	40
	Anneal/Extend	60	30 sec	
7900HT Fast	AmpliTaq® Fast DNA Polymerase, UP Activation	95	20 sec	HOLD
	Denature	95	1 sec	40
	Anneal/Extend	60	20 sec	

Thermopol cycling condition:

Step	Temp	Time
Denaturation	95°C	30 seconds
35 Cycles	95°C	30 seconds
	60°C	30 seconds
	68°C	30 seconds
Final extension	68°C	5 minutes
Hold	4°C	

SYBR Green

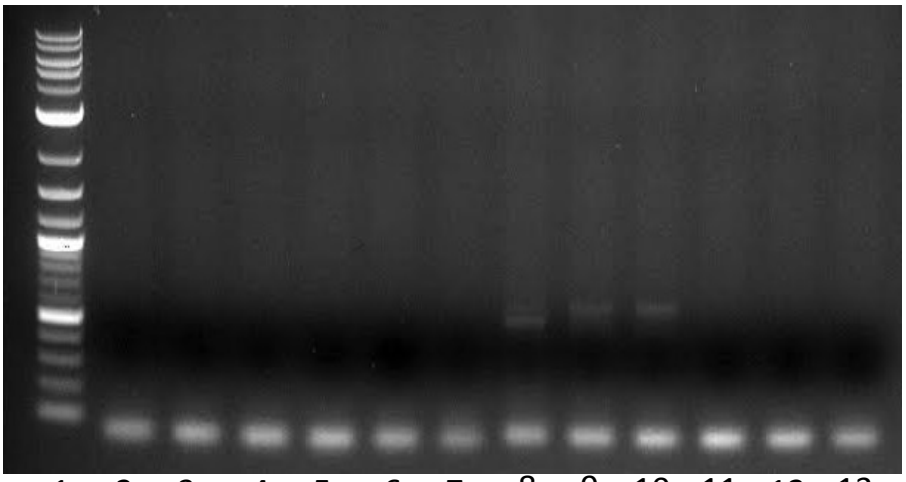


1 - 2 log ladder
2,8 - 53°C
3,9 - 55°C
4,10 - 57°C
5,11 - 60°C
6,12 - 62°C
7,13 - 64°C

1 2 3 4 5 6 7 8 9 10 11 12 13

Ahd1 RBOFOX3

Thermopol



1 - 2 log ladder
2,8 - 53°C
3,9 - 55°C
4,10 - 57°C
5,11 - 60°C
6,12 - 62°C
7,13 - 64°C

1 2 3 4 5 6 7 8 9 10 11 12 13

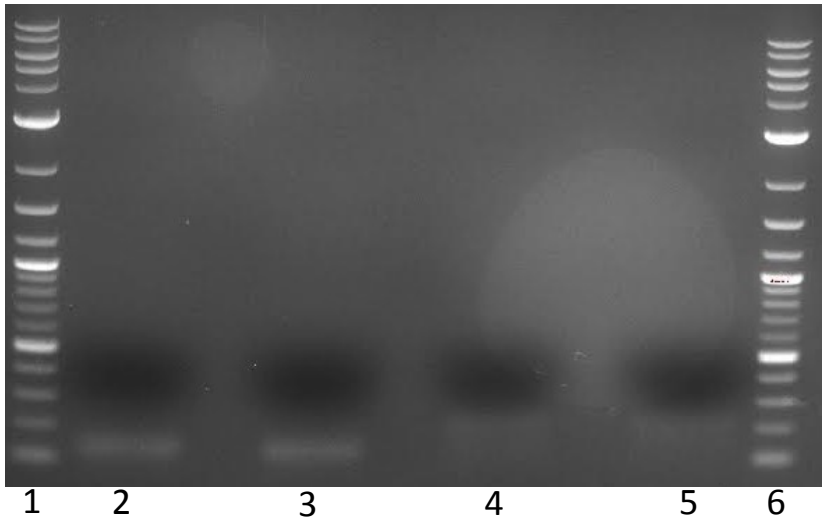
Ahd1 RBOFOX3

10ng of ChIP DNA used. 30 µl reaction volume.

		Final conc.	µl
SYBR Green	2X	1x	15
Primer	2000nM	200nM	10
DNA			10
Total			30

Fast SYBR green thermal cycling condition:

Select Fast Mode				
Instrument	**Step**	**Temperature (°C)**	**Duration**	**Cycles**
• Step One • StepOne Plus • 7500 Fast	AmpliTaq® Fast DNA Polymerase, UP Activation	95	20 sec	HOLD
	Denature	95	3 sec	40
	Anneal/Extend	60	30 sec	
7900HT Fast	AmpliTaq® Fast DNA Polymerase, UP Activation	95	20 sec	HOLD
	Denature	95	1 sec	40
	Anneal/Extend	60	20 sec	



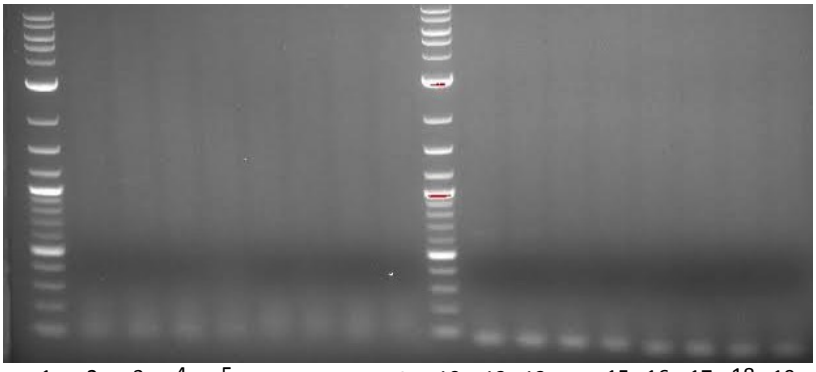
1,6 - 2 log ladder
2,3 – RBFOX3
4,5 – ADH1

Objective: Determining optimal annealing temperature
400ng of DNA used for amplification. Please note that the annealing temperature tested were 55°C, 57°C, 60°C, 63°C.
Fast SYBR green thermal cycling condition:

Select Fast Mode				
Instrument	**Step**	**Temperature (°C)**	**Duration**	**Cycles**
• Step One • StepOne Plus • 7500 Fast	AmpliTaq® Fast DNA Polymerase, UP Activation	95	20 sec	HOLD
	Denature	95	3 sec	40
	Anneal/Extend	60	30 sec	
7900HT Fast	AmpliTaq® Fast DNA Polymerase, UP Activation	95	20 sec	HOLD
	Denature	95	1 sec	40
	Anneal/Extend	60	20 sec	

Thermopol cycling condition:

Step	Temp	Time
Denaturation	95°C	30 seconds
35 Cycles	95°C	30 seconds
	60°C	30 seconds
	68°C	30 seconds
Final extension	68°C	5 minutes
Hold	4°C	



1, 10 - 2 log ladder
2,6,12,16 - 55°C
3,7,13,17 - 57°C
4,8,14,18 - 60°C
5,9,15,19 - 63°C

Objective: Titration of RBFOX3 and ADH1 primers for qPCR.
200 ng of DNA used for amplification.

Primer conc.	nM	400	200	100	50
nM	400	400/400	200/400	100/400	50/400
nM	200	400/200	200/200	100/200	50/200
nM	100	400/100	200/100	100/100	50/100
nM	50	400/50	200/50	100/50	50/50

Primer stock	2µM							
primer conc.(nM)	400/400	200/400	100/400		primer conc.(nM)	400/400	200/400	100/400
	µl	µl	µl			µl	µl	µl
SYBR green	10	10	10		SYBR green	30	30	30
Forward primer	4	2	1		Forward primer	12	6	3
Reverse primer	4	4	4		Reverse primer	12	12	12
DNA	2	2	2		DNA	6	6	6
H2O	0	2	3		H2O	0	6	9
Total	20	20	20		Total	60	60	60
primer conc.(nM)	400/200	200/200	100/200		primer conc.(nM)	400/200	200/200	100/200
	µl	µl	µl			µl	µl	µl
SYBR green	10	10	10		SYBR green	30	30	30
Forward primer	4	2	1		Forward primer	12	6	3
Reverse primer	2	2	2		Reverse primer	6	6	6
DNA	2	2	2		DNA	6	6	6
H2O	2	4	5		H2O	6	12	15
Total	20	20	20		Total	60	60	60
primer conc.(nM)	400/100	200/100	100/100		primer conc.(nM)	400/100	200/100	100/100
	µl	µl	µl			µl	µl	µl
SYBR green	10	10	10		SYBR green	30	30	30
Forward primer	4	2	1		Forward primer	12	6	3
Reverse primer	1	1	1		Reverse primer	3	3	3
DNA	2	2	2		DNA	6	6	6
H2O	3	5	6		H2O	9	15	18
Total	20	20	20		Total	60	60	60
primer conc.(nM)	400/50	200/50	100/50		primer conc.(nM)	400/50	200/50	100/50
	µl	µl	µl			µl	µl	µl
SYBR green	10	10	10		SYBR green	30	30	30
Forward primer	4	2	1		Forward primer	12	6	3
Reverse primer	0.5	0.5	0.5		Reverse primer	1.5	1.5	1.5
DNA	2	2	2		DNA	6	6	6
H2O	3.5	5.5	6.5		H2O	10.5	16.5	19.5
Total	20	20	20		Total	60	60	60

primer conc.(nM)	400/400	200/400	100/400		primer conc.(nM)	400/400	200/400	100/400
	µl	µl	µl			µl	µl	µl
Thermopol master mix	2	2	2		Thermopol master mix	8	8	8
Forward primer	4	2	1		Forward primer	16	8	4
Reverse primer	4	4	4		Reverse primer	16	16	16
dNTP	0.5	0.5	0.5		dNTP	2	2	2
Template	2	2	2		Template	8	8	8
Polymerase	0.125	0.125	0.125		Polymerase	0.5	0.5	0.5
H2O	7.38	9.38	10.38		H2O	29.5	37.5	41.5
Total	20	20	20		Total	80	80	80
primer conc.(nM)	400/200	200/200	100/200		primer conc.(nM)	400/200	200/200	100/200
	µl	µl	µl			µl	µl	µl
Thermopol master mix	2	2	2		Thermopol master mix	8	8	8
Forward primer	4	2	1		Forward primer	16	8	4
Reverse primer	2	2	2		Reverse primer	8	8	8
dNTP	0.5	0.5	0.5		dNTP	2	2	2
Template	2	2	2		Template	8	8	8
Polymerase	0.125	0.125	0.125		Polymerase	0.5	0.5	0.5
H2O	9.38	11.38	12.38		H2O	37.5	45.5	49.5
Total	20	20	20		Total	80	80	80
primer conc.(nM)	400/100	200/100	100/100		primer conc.(nM)	400/100	200/100	100/100
	µl	µl	µl			µl	µl	µl
Thermopol master mix	2	2	2		Thermopol master mix	8	8	8
Forward primer	4	2	1		Forward primer	16	8	4
Reverse primer	1	1	1		Reverse primer	4	4	4
dNTP	0.5	0.5	0.5		dNTP	2	2	2
Template	2	2	2		Template	8	8	8
Polymerase	0.125	0.125	0.125		Polymerase	0.5	0.5	0.5
H2O	10.38	12.38	13.38		H2O	41.5	49.5	53.5
Total	20	20	20		Total	80	80	80
primer conc.(nM)	400/50	200/50	100/50		primer conc.(nM)	400/50	200/50	100/50
	µl	µl	µl			µl	µl	µl
Thermopol master mix	2	2	2		Thermopol master mix	8	8	8
Forward primer	4	2	1		Forward primer	16	8	4
Reverse primer	0.5	0.5	0.5		Reverse primer	2	2	2
dNTP	0.5	0.5	0.5		dNTP	2	2	2
Template	2	2	2		Template	8	8	8
Polymerase	0.125	0.125	0.125		Polymerase	0.5	0.5	0.5
H2O	10.88	12.88	13.88		H2O	43.5	51.5	55.5
Total	20	20	20		Total	80	80	80

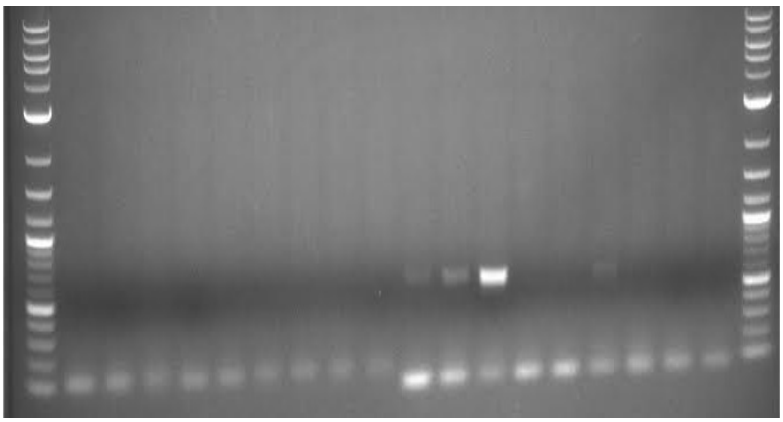
Fast SYBR green thermal cycling condition:

Select Fast Mode				
Instrument	**Step**	**Temperature (°C)**	**Duration**	**Cycles**
• Step One • StepOne Plus • 7500 Fast	AmpliTaq® Fast DNA Polymerase, UP Activation	95	20 sec	HOLD
	Denature	95	3 sec	40
	Anneal/Extend	60	30 sec	
7900HT Fast	AmpliTaq® Fast DNA Polymerase, UP Activation	95	20 sec	HOLD
	Denature	95	1 sec	40
	Anneal/Extend	60	20 sec	

Thermopol cycling condition:

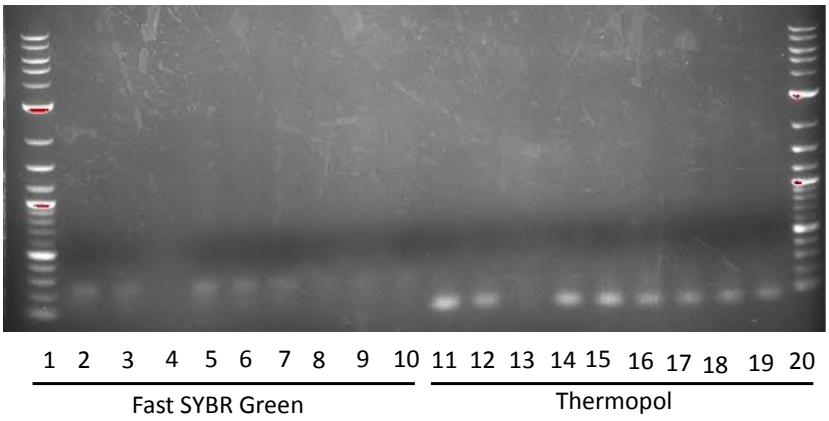
Step	Temp	Time
Denaturation	95°C	30 seconds
35 Cycles	95°C	30 seconds
	60°C	30 seconds
	68°C	30 seconds
Final extension	68°C	5 minutes
Hold	4°C	

RBFOX3



1, 20 - 2 log ladder
2,11 - 400nM For/ 400nM Rev
3, 12 - 200nM For/ 400nM Rev
4, 13 - 100nM For/ 400nM Rev
5, 14 - 400nM For/ 200nM Rev
6, 15 - 200nM For/ 200nM Rev
7, 16 - 100nM For/ 200nM Rev
8, 17 - 400nM For/ 100nM Rev
9, 18 - 200nM For/ 100nM Rev
10,19 - 100nM For/ 100nM Rev

Adh1



1, 20 - 2 log ladder
2,11 - 400nM For/ 400nM Rev
3, 12 - 200nM For/ 400nM Rev
4, 13 - 100nM For/ 400nM Rev
5, 14 - 400nM For/ 200nM Rev
6, 15 - 200nM For/ 200nM Rev
7, 16 - 100nM For/ 200nM Rev
8, 17 - 400nM For/ 100nM Rev
9, 18 - 200nM For/ 100nM Rev
10,19 - 100nM For/ 100nM Rev

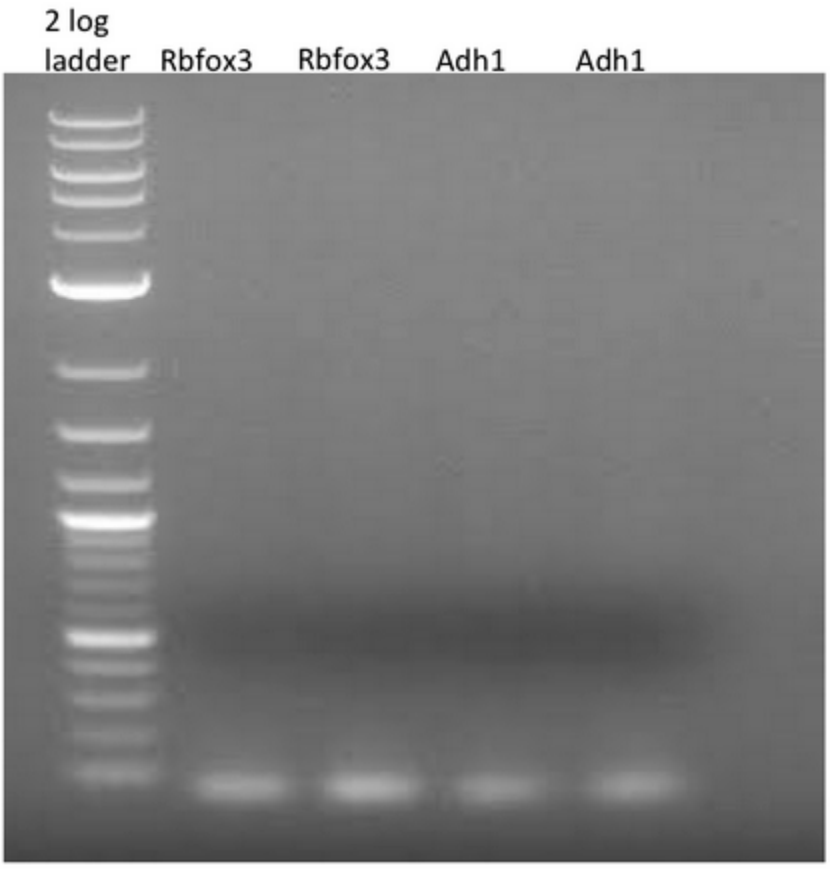
Using SYBR Green Universal Master Mix

	Final conc.	µl
2X SYBR green PCR master mix	1x	12.5
Primer (5µM)	200 nM	1
Template		2
Water		9.5

Total		25

Step	AmpliTaq Gold® Polymerase Activation†	PCR	
	HOLD	CYCLE (40 cycles)	
		Denature	Anneal/Extend
Temp.	95.0 °C	95.0 °C	60.0 °C
Time	10 min	15 sec	1 min
Volume	50 µL		

† The 10 min, 95°C step is required to activate the AmpliTaq Gold® DNA Polymerase.



Conclusions: These data show single bands in each experimental well, indicating a specific product has been obtained of the correct size for each gene and that the PCR conditions have been optimized.

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